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DEPOSITION OF CUTICULAR SUBSTANCES *IN VITRO* BY LEG REGENERATES FROM THE COCKROACH, *LEUCOPHAEA MADERAE* (F.)

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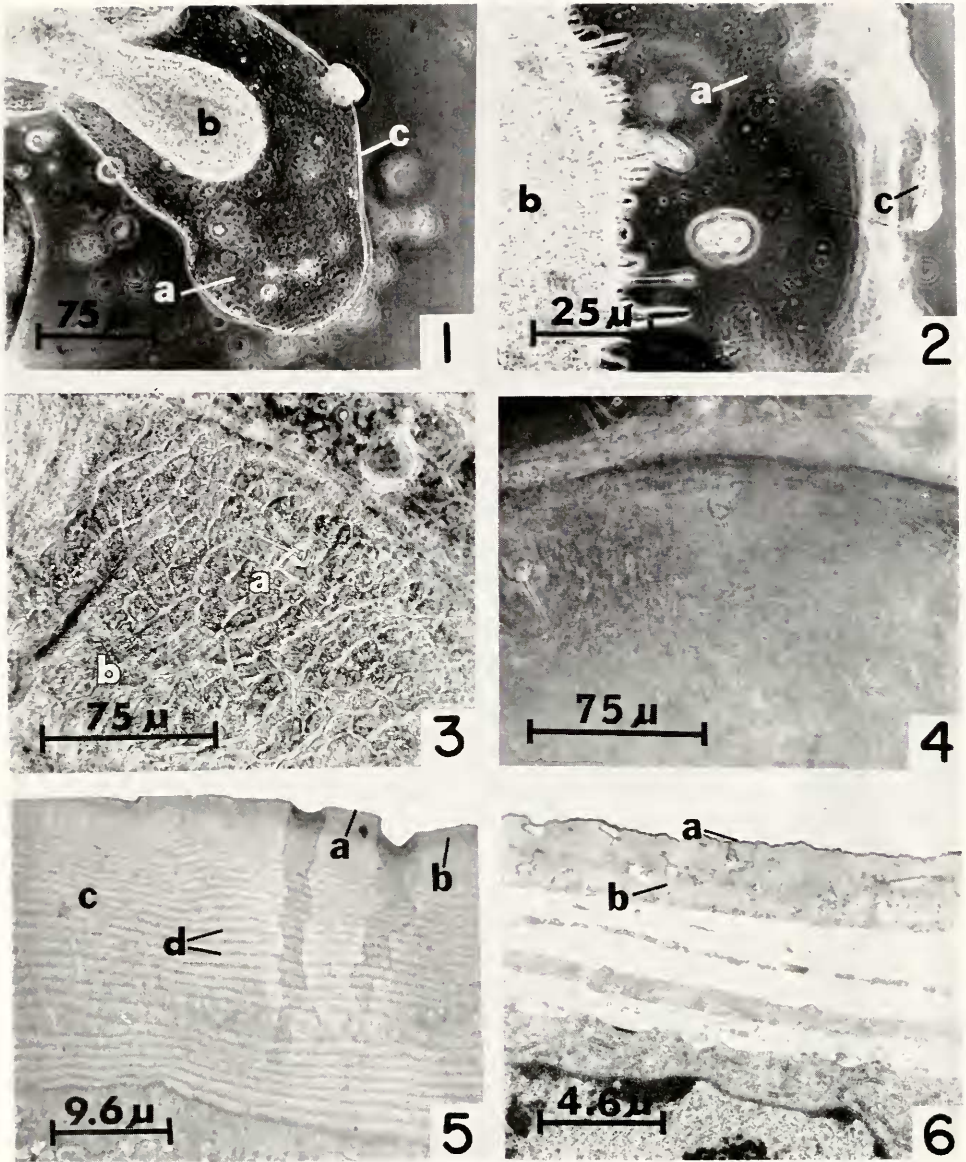
The appearance of cuticle-like membranes on the surface of insect tissues maintained *in vitro* has been reported by several investigators. Demal (1956) found a thin membrane covering explants of leg imaginal discs from 3-hour-old *Drosophila* prepupae held *in vitro*. He also observed thickening of the cuticle and the formation of tarsal claws. Sengal and Mandaron (1969) explanted leg imaginal discs of late-instar *Drosophila* larvae with the cephalic complex (including the ring gland); when the cultures were treated with ecdysone, many leg discs showed morphogenic changes that included the appearance of setae, surface scultpturing, and claws. Marks and Reinecke (1964) reported the secretion of a gelatinous material on the epidermis of leg regenerates of the cockroach, *Leucophaea maderae* (Fabr.), and Larsen (1966) cultured heart fragments from cockroach embryos and found a cuticle-like membrane covering vesicles that formed on these explants. Miciarelli Sbrenna, and Colombo (1967), working with explants of body wall from the abdomen of nymphs of the locust, *Schistocerca gregaria* (Forsk), found that the developmental stage of the donor insect greatly influenced the deposition of cuticle. Agui, Yagi, and Fukaya (1969) treated explants of epidermis from diapausing larvae of *Chilo suppressalis* (Walker) by adding small amounts of ecdysterone to the culture medium, and within 48 hours, the explant shed its old cuticle and deposited a new one.

Ritter and Bray (1968) cultured clots of blood from the cockroach, *Gramphadorina portentosa* in a medium that was free of insect plasma. The subcultures developed strands, flakes, and platelets around and within the clot, and the investigators concluded that the deposits were made up of a protein-chitin complex and that the complex was deposited on the surface of the culture vessel. However, the electron micrographic studies of Locke (1964) indicated that at least *in vivo,* the precursors are secreted by the epidermal cells.

Deposition of the cuticle in the regenerating leg of nymphs of the cockroach, *L. maderae*, was studied *in vivo* by Leopold and Marks (unpublished observations), and they found that two sheaths formed during regeneration. One appeared early in the development of the regenerate and thickened to form a gelatinous envelope around the regenerate. This layer apparently consisted of a protein-carbohydrate complex. The second sheath contained chitin, appeared late in development, thickened, and became wrinkled and rugose. The initial sheath was sloughed before molting, and the underlying chitin-bearing layer became the functional cuticle.



FIGURES 1–6.

FIGURE 1. A gelatinous sheath (a) laid down by the explant (b). The sharply defined outer border (c) is occasionally found in old, untreated cultures: *in vivo* 35 days: *in vitro* 53 days.

FIGURE 2. Gelatinous sheath (a) secreted by untreated explant (b) shows epithelial cells (c) extending into the sheath: *in vivo* 25 days; *in vitro* 40 days.

FIGURE 3. Detail of the surface of a mature leg regenerate that developed spontaneously *in vitro*. Seta (a) and surface sculpture (b) are characteristic: *in vivo* 35 days; *in vitro* 28 days.

In the present study, we have investigated some of the processes involved in cuticle secretion by combining the leg regenerate system with an *in vitro* methodology and histochemical analysis.

Materials and Methods

Cockroach leg regenerates of different ages were prepared as described by Marks (1968). In the first tests, leg regenerates allowed to develop eight days after leg removal were dissected and placed under dialysis strips in Rose chambers with glass coverslips. The chemically defined M-7 medium was used. Six days later, they were examined with a phase contrast microscope, and the chambers were refilled with medium containing the test substance. After six more days, they were re-examined, and those showing cuticle-like deposits were scored as positive.

In the second test, leg regenerates were allowed to develop from 10 to 35 days after leg removal and were then dissected and examined. Those showing evidence of a cuticle were discarded, and the remainder were placed in Rose chambers between the dialysis strip and a glass coverslip. A plastic coverslip was added, and the chambers were filled with M-18 culture medium to which 5% fetal calf serum was added. The test was arranged with pairs of chambers, each containing a pair of leg regenerates from opposite sides of a single cockroach. One chamber of each pair was treated with ecdysterone, and the other was used as the control. Some of the controls were completely untreated, and others were treated with the inactive analog 22-iso-ecdysone (kindly supplied by Dr. John Siddall, Zoecon Corp., Palo Alto, California). The pairs were examined daily with a phase contrast microscope, and differences between the treated and untreated chambers were recorded. If the control showed evidence of developing cuticle, the treated chamber paired with it was removed from consideration. After 15 days, pairs in which the test leg developed a cuticular deposit were separated, and the test leg was discarded. Then the control was given 5 to 10 μg of ecdysterone to prove that its failure to produce cuticle had occurred because of the absence of the hormone and not from other causes.

The presence of chitin in selected specimens was verified by using the fluorescent enzyme technique of Benjaminson (1969). Frozen sections of the leg regenerate tissue were treated with chitinase conjugated with the fluorescent dye lissamine rhodamine B 200 chloride. They were examined by fluorescence microscopy, and fluorescence in cuticular structures was accepted as evidence of chitin.

Results and Discussion

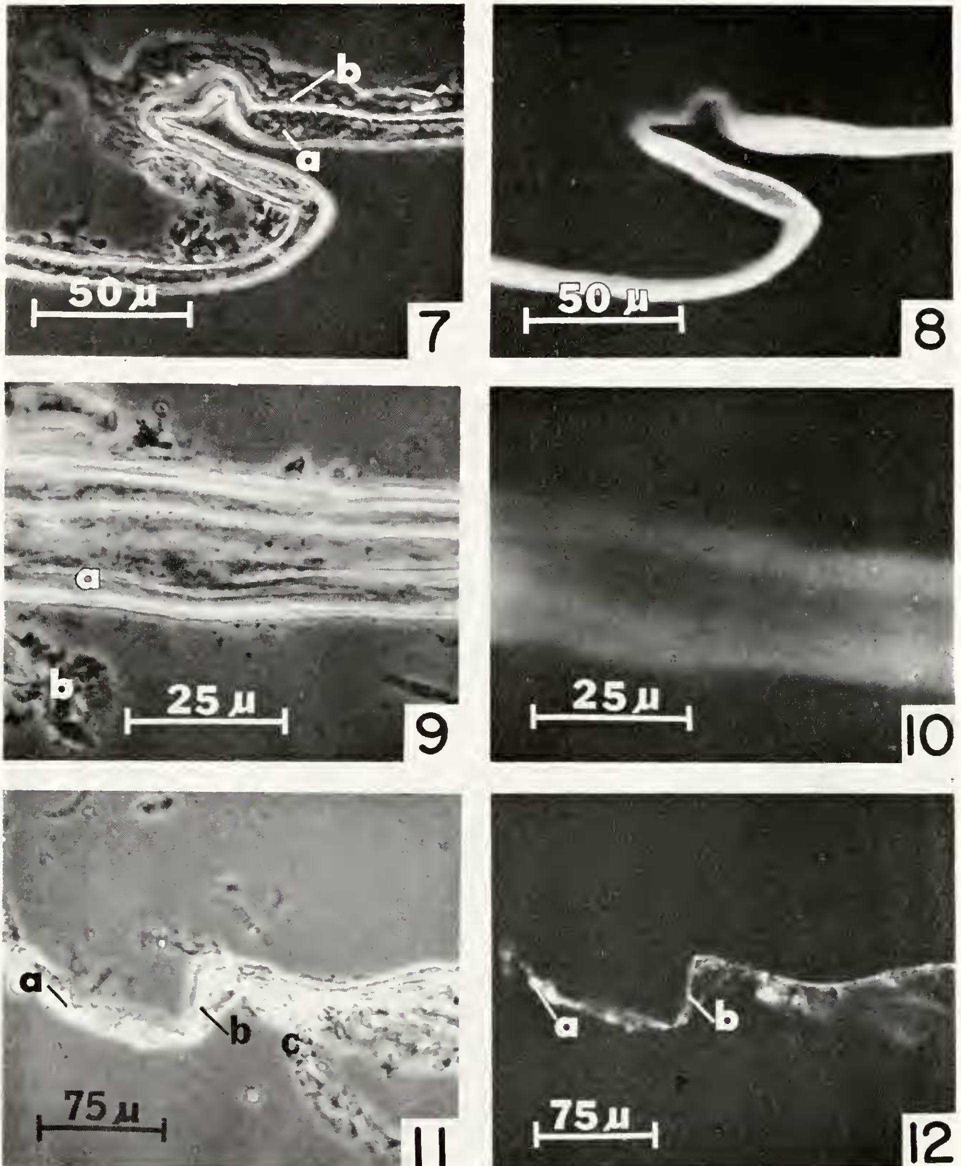
Deposition of the sheath

Leg regenerates that were allowed to develop for eight days before explanation normally showed no cuticle-like deposits when they were explanted. However,

Figure 4. Detail of the surface of a 10-day-old leg regenerate treated with 12 μg/ml of ecdysterone. Setae are present but surface sculpture is absent: *in vivo* 10 days; *in vitro* 18 days.

Figure 5. Electron micrograph of cuticle from the leg of a newly molted cockroach. Epicuticle (a), sclerotized layer (b), and endocuticle (c) are clearly visible. Laminae (d) of the endocuticle are well formed and compact.

Figure 6. Electron micrograph of cuticle formed by a 25-day-old leg regenerate *in vitro* shows well-formed epicuticle (a) but poorly formed endocuticle (b): *in vivo* 25 days; *in vitro* 30 days.



FIGURES 7–12.

FIGURE 7. A section through cuticle from leg of freshly molted cockroach. This specimen is shown under phase contrast illumination. Cuticle (a), which is refractible, is underlain by the epidermis (b).

FIGURE 8. Same specimen as figure 7 subjected to Benjaminson chitinase procedure and fluorescent microscopy. The chitinase-dye complex that has conjugated with the cuticle fluoresces. Note that the epidermis does not conjugate with the enzyme-dye complex and is no longer visible.

when they were held *in vitro*, 13 per cent produced deposits of gelatinous material within 14 days. The deposit appeared as a thin transparent layer that gradually thickened (Figs. 1 and 2); also, flakes and threads of darker material and occasional layering of this material appeared. As the cultures aged, the outer border of the layer often became sharply defined and yellowish (Fig. 1). No evidence of definitive cuticle appeared even though some cultures were held as much as 120 days. Fluorescent chitinase tests showed that the gelatinous layer, which probably represented the protective sheath seen in *in vivo* preparations, was devoid of chitin.

The protective role of this sheath, which may be related to wound cuticle (Sannasi, 1968), is supported by the finding that methanol (0.5 μl/ml of medium) and incubates of muscle tissue were as effective in inducing sheath deposition as were ecdysterone (2.5 μg/ml of medium) and incubates of the prothoracic gland. This sheath material is probably the same as the gelatinous substance reported by Marks and Reinecke (1964) and may also be the same as the cuticular material reported by Larsen (1966).

Deposition of the cuticle

The first evidence of the deposition of the cuticle *in vitro* was the appearance of a thin refractile layer between the epidermal cells and the protective sheath. As the deposition continued, the epidermal cells rounded up, contracted, and produced a pebbled appearance. Parallel ridges appeared and increased in size and number so the entire surface of the explant had a rugose appearance. Setae formed from hair-like trichogen cells that protruded from the explant, and the tormagen cell became embedded in the surface and sclerotized (Figs. 3 and 4). The deposition of the cuticle was usually terminated by the withdrawal of the epidermal cells from the secreted structures and the development of a space between them. This process apparently represents the *in vitro* counterpart of apolysis.

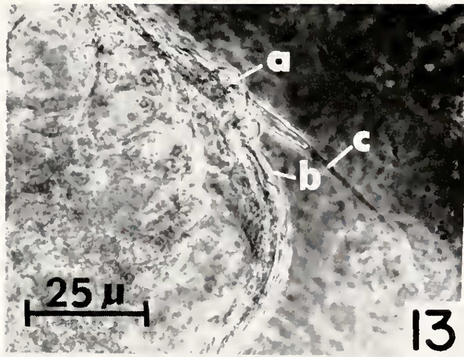
Examination of the ultrastructure showed that the cuticle regenerated *in vivo* possessed a thick endocuticle at the time of molting, and that the lamellae were compact and well formed (Fig. 5). Cuticle that formed *in vitro* (Fig. 6) showed a well developed epicuticle, but the endocuticle was only partially formed and not as compact as cuticle formed *in vivo*; separation of the cuticle from the epidermis was apparent.

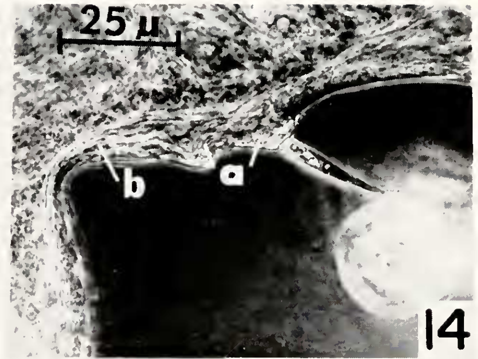
FIGURE 9. Section through cuticle (a) formed by a 25-day-old leg regenerate *in vitro* seen with phase contrast illumination. Epidermis (b) has been largely stripped away in sectioning. The membrane is folded so the two layers are visible: *in vivo* 25 days; *in vitro* 20 days.

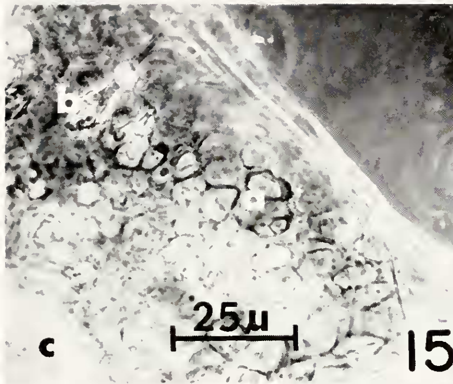
FIGURE 10. The same specimen as in Figure 9 subjected to the Benjaminson chitinase procedure and viewed by fluorescent microscopy shows two layers of fluorescence. Note fluorescence is not as intense as in Figure 8.

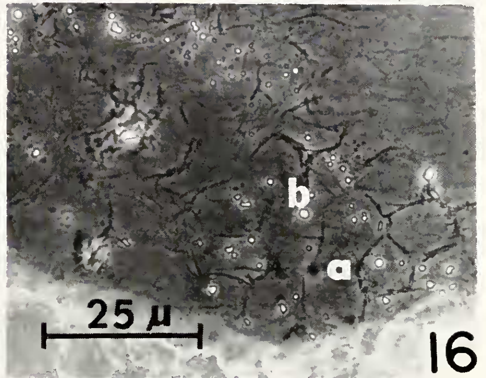
FIGURE 11. A section through cuticle formed *in vitro* by a 10-day-old leg regenerate viewed under phase contrast illumination. Note refractile droplets (a) appearing between the cuticle (b) and epidermis (c): *in vivo* 10 days; *in vitro* 21 days.

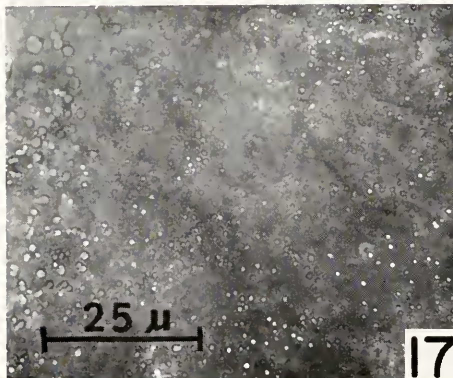
FIGURE 12. Same specimen as in Figure 11 subjected to the Benjaminson chitinase procedure and viewed by fluorescent microscopy. Both droplets (a) and solid cuticle (b) fluoresce, indicating the presence of chitin.

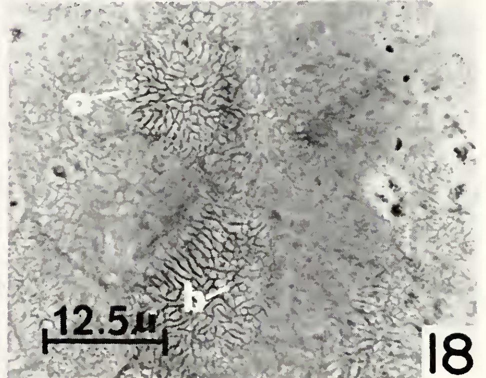












FIGURES 13–18.

FIGURE 13. A leg regenerate treated with 5 µg/ml of ecdysterone shows seta formation. Base of seta lies in the socket (a) that protrudes from the surface of the cuticle (b). The unsclerotized tip of the trichogen cell (c) is visible: *in vivo* 25 days; *in vitro* 25 days.

FIGURE 14. Another area of the same leg regenerate shown in Figure 13 shows two setae with well-formed sockets (a) embedded in cuticle (b). Only the base of the seta is sclerotized; upper end remains membranous: *in vivo* 25 days; *in vitro* 25 days.

FIGURE 15. Reticular pattern (a) and abnormal setae (b) are present on the surface of cuticle developing *in vitro*. Epidermis (c) has withdrawn, leaving the cuticle behind: *in vivo* 25 days; *in vitro* 25 days.

The dense cuticle formed *in vivo* demonstrated a strong reaction for chitin (Figs. 7 and 8) when it was tested by the fluorescent chitinase procedure. When the cuticle that formed *in vitro* was tested by this method, the reaction was weak but consistently positive (Figs. 9 and 10). The lower intensity of the fluorescence probably resulted from the lower density of the endocuticle in the *in vitro* preparations.

In some specimens, particularly among the younger explants, droplets of hyaline material often accumulated between the developing cuticle and the surface of the epithelial cells. When one such explant was sectioned and tested for chitin, we found a pebbled inner surface that contained chitin in the form of droplets (Figs. 11 and 12). Such droplets appeared only after treatment with ecdysterone and were most commonly found when the dosage was very low. The development of setae *in vitro* occurred with greatest frequency in explants that were taken from older nymphs (Table I). In some cases, the socket formed on the surface of the cuticle (Fig. 13) rather than imbedded in it (Fig. 14), and generally the sclerotization was incomplete.

We occasionally found cuticle forming over the surface of a vesicle, and when the vesicle later collapsed and withdrew the delicate epithelium, the remaining cuticle was left with a pattern representing the outline of the cells that deposited it (Figs. 15 and 16). The presence of the hyaline droplets often made it difficult to observe these patterns, but in those areas where they were absent, a pattern of ridges within the area laid down by a single cell was seen (Fig. 18). This apparently represented the imprint of the surface of the cell itself.

When leg regenerates were dissected late in the molting cycle (35 days), a large number had already initiated cuticle development *in vivo*. Those that had not were placed *in vitro*, and more than half continued their development without stimulation (Table I). Therefore, meaningful studies of the induction of cuticle development could only be made by using paired chambers containing legs from opposite sides of the same insect.

In an attempt to simplify our procedures and to increase our efficiency, we made a study of younger leg regenerates. Paired chambers were used, and leg regenerates from 5- to 30-day-old were treated with different doses of ecdysterone. The results indicated that while all doses above 2 μg/ml had about the same effect on cuticle production, the frequency with which the leg regenerates responded to stimulation increased with age (*in vivo*) until a 100 per cent response was reached at 20 days. There was also a corresponding increase in the frequency of seta formation with age.

The increase in ability to respond to the hormone with increased age seemed to continue after the tissue was placed *in vitro*. Thus, 10-day-old legs treated

FIGURE 16. Reticular pattern (a) is visible in this very thin cuticle. Droplets (b) probably containing chitin (Figure 11) are present. Epidermis has withdrawn: *in vivo* 25 days; *in vitro* 10 days.

FIGURE 17. Detail of the surface of a developing cuticle shows droplets accumulating between the cuticle and the epidermis. Droplets probably contain chitin; *in vivo* 10 days; *in vitro* 18 days.

FIGURE 18. Surface detail of developing cuticle shows polygonal outlines of cells (a). Within the outlines is a pattern (b) that represents the negative image of the irregular surface of the cell. This may represent the first step in the formation of the surface sculpture: *in vivo* 25 days; *in vitro* 10 days.

four days after explanation gave a 20 per cent (2/10) response, but when the water controls for this series were treated with ecdysone 14 days later, there was a 66 per cent (4/6) response. A similar but less marked effect was obtained with 15-day-old leg regenerates. The untreated controls did not produce cuticle.

A second test was set up to determine the minimum dose of ecdysterone that would produce a 100 per cent response in 25-day-old regenerates. Paired chambers were not used because regenerates of this age did not spontaneously produce cuticle under experimental conditions. In the 12 specimens treated with 2 μl of water and in the 10 specimens treated with 24 μg/ml of 22-iso-ecdysterone, there was no development of cuticle; all specimens developed cuticle when they were later treated with 10 μg/ml of ecdysterone for 25 hours. In the experimental chambers, doses of ecdysterone in water ranging from 2.5 μg/ml of nutrient to 0.05 μg/ml were given; then after seven days, the chambers were emptied and refilled with fresh nutrient, and the date when cuticle deposition

TABLE I

The effect of age at time of explanatation on development of cuticle in cockroach leg regenerates in vitro

Days after leg removal	Number tested	Per cent developing without treatment	Number (and per cent) developing after treatment with 2–10 μg/ml ecdysterone	
			Cuticle	Setae
10	10	0	2 (20)	1 (10)
15	8	0	7 (88)	1 (12)
20	6	0	6 (100)	2 (33)
25	16	0	16 (100)	10 (62)
30	4	0	4 (100)	3 (75)
35	5 (11)*	54	5 (100)	3 (60)

* Eleven were set up, and six (54%) developed spontaneously. All five remaining regenerates developed cuticle when treated with ecdysterone.

first appeared was recorded. A 100 per cent response was obtained with 2.5 μg/ml, a 90 per cent response was obtained with doses down to 0.5 μg/ml, a 37 per cent response was obtained with a dose of 0.2 μg/ml, and no response was obtained with a dose of 0.1 μg/ml or less. All the controls that were later exposed to the hormone developed cuticle.

The appearance of setae and surface sculpture and the presence of chitin indicated that the cuticular deposits formed *in vitro* in response to stimulation by ecdysterone are the same as those deposits that are present *in vivo* at the time of molting. Thus, it is likely that the structures reported by Demal (1956) and Sengel and Mandaron (1969) as appearing on imaginal leg discs taken from late-instar *Drosophila* larvae probably also contained chitin. In Demal's studies, development was spontaneous since stimulation occurred before the leg imaginal discs were explanted, but evaluation of the results of Sengel and Mandaron is more difficult because the brain and ring gland were present in the same cultures as the imaginal discs and because development occurred in some of the carrier-control cultures. Interaction between the glands and the ecdysone cannot be ruled out since Burdette, Hanley, and Grosch (1968) reported that the effect

of ecdysone on the ocular imaginal discs of *Drosophila* was enhanced by the presence of the cephalic lobes in the culture, and Williams (1952) showed that ecdysone can stimulate secretion by the prothoracic glands. However, the amount of ecdysone used by Sengel and Mandaron in the culture medium would probably have been sufficient to induce cuticle secretion by the leg imaginal discs, even in the absence of the gland explants. The work of Agui *et al.* (1969) further demonstrated that the entire process of molting, including the shedding of the old cuticle and the deposition of the new, can be induced in short-term cultures by adding ecdysterone to the culture medium.

In leg regenerates, the deposition of endocuticle *in vitro* is usually incomplete and varies considerably from one specimen to another. In general, the 35-day-old leg regenerates that produced cuticle *in vitro* tended to produce heavier deposits than did the 10- to 20-day-old ones. Two explanations for this are offered: The *in vitro* conditions may have been such that the normal synthesis of the endocuticle by the epithelial cells could not occur; therefore, the cuticle secreted *in vitro* would consist primarily of the epicuticle and that portion of the endocuticle secreted before the synthesis mechanism ceased to function. The other possibility, suggested by the experiments of Philogene and McFarlane (1967), is that the epithelial cells merely lay down a material that is either produced elsewhere or whose precursors are produced elsewhere. Then, by isolating the leg regenerate, we cut off the source of supply of this material except for that amount explanted along with the leg regenerate. This amount would be greater in regenerates explanted later in the molting cycle.

The fact that a 24-hour exposure of the leg regenerate to 10 μg/ml of ecdysterone was sufficient to induce cuticle deposition a week later suggests that the so-called "endogenous hormone" present in explants taken from insects late in the molting cycle probably refers to the physiological state of the tissue rather than to the actual presence of the hormone in the tissue. The fact that 10-day-old leg regenerates (which are little more than blood-filled sacs of epithelial tissue) can be induced to produce a seta-bearing cuticle with relatively small doses of ecdysterone indicates that this physiological state depends on the action of the hormone as well as on the age of the tissue. In *Leucophaea*, removal of a leg late in the stadium when the ecdysone titer is presumably high resulted in the formation of a cuticle-covered papilla, and regeneration of the leg resumed only after the subsequent molt. This *in vivo* response closely resembeled the response produced *in vitro* by treatment of 10-day-old leg regenerates with ecdysterone in which premature cuticle deposition eventually terminated morphogenesis.

However, if the leg was removed earlier in the cycle and allowed a longer period of development, a complete leg formed, and the approach of molting merely speeded up and completed the process. Similarly, Postlethwait and Schneiderman (1970) showed that when leg imaginal discs of *Drosophila* cultured *in vivo* were treated with large doses of ecdysterone, metamorphosis complete with formation of pupal cuticle occurred.

In our studies, we found that cuticle deposition can be stimulated by small doses of hormone presumably because other tissues (blood and fat body) are not present to inactivate the residual hormone and the target tissue is thus exposed to low levels of the hormone over a period of several days. In *in vivo* cultures, very large doses of the hormone were required to produce such an effect, thus

suggesting that there may be a relationship between the amount of hormone present and the length of time that the tissue is exposed to it.

Ohtaki, Milkman and Williams (1968) proposed that tissue competence results from the accumulation of physiological events caused by continuous exposure to a low titer of hormone over a long period. It is just such a situation that we reproduced *in vitro, i.e.,* a low dose (0.2 μg/ml) of hormone left in contact with the target organ over a long period (7 days). Our results tend to substantiate the hypothesis of Williams' group. Furthermore, it should be possible to use this experimental system as a basis for a series of time-dosage studies that would shed additional light on this question.

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Summary

Two types of sheath material are deposited during the development of leg regenerates in the cockroach, *L. maderae.* The first sheath to appear resembles wound cuticle because it contains no chitin. Its deposition *in vitro* can be induced by a number of unrelated substances. The second sheath contains chitin, bears spines and setae, and represents the cuticle. Deposition of this sheath is induced *in vitro* by microgram quantities of ecdysterone but not by other substances that were tested.

Leg regenerates dissected as early as 10 days after leg removal were capable of depositing cuticle, but a 100% response to stimulation with ecdysterone was obtained only with leg regenerates 20 or more days old.

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